DETERMINATION OF LYSIS ABILITY AND LOCATION OF SUSPECTED TAIL LYSIN GP156 OF PHAGE K AGAINST *STAPHYLOCOCCUS AUREUS*

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Determination of Lysis Ability and Location of Suspected Tail Lysin gp156 of Phage K Against *Staphylococcus aureus*

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As the issue of bacterial antibiotic resistance becomes more prevalent, phage technology research attempts to investigate viruses to possibly target and kill pathogens effectively with specificity. This study is an investigation into the product of gene *156* (gp156) of phage K, to determine its location in the phage structure and the extent of its lysis ability. Through previous experiments with temperature sensitive (*ts*) mutants in our lab, we have determined gene *156* to be essential to the phage's reproduction. Through genome sequencing of phage K's characteristically large genome, gene *156* is found to be located in the tail-structural module next to gene *157*, a studied tail lysin gene. This investigation will attempt to clone genes *156* & *157* as PCR products with specially designed primers into pETDuet-1 in order to isolate and express these genes. Further study of the gene products activity will be conducted separately and in combination. SDS-PAGE gels will aid in determining if gp156 is a part of the virion. Stability assays and potassium release data will indicate the role that gp156 plays in the viral structure and injection of DNA into its host. The conclusions from this study would be beneficial in future studies of phages related to phage K and potentially inform bactericidal alternatives to antibiotic resistant *S. aureus* and beyond.

DEDICATION

This project is dedicated to my fellow peers who try the best they can to do good. This is for people who better their life and the lives of others. I also dedicate this to my past educators who have given me unconditional patience, as I will always honor their efforts and sacrifices by doing the best I can.

ACKNOWLEDGEMENTS

Throughout my research experience, I learnt that advancement in science takes a dedicated, passionate team. I am also moved by the devotion of Dr. Gill and Dr. Leavitt to helping me build my foundations into research. I want to thank them for their long hours and patience, allowing me to learn, make mistakes, and grow as a scientist on their dime. This project is meaningful to me beyond science, as it taught me more about my strength and limits. Thanks to my mentors, I am now more informed of myself and how to be like the highly-effective people I have had the pleasure of learning from. This project, however, comes with a sacrifice of time with family and close friends, and the love of my life. I love my Mom, Dad, Sister, and Brother, and I would be no where without their love, lessons, and support. I would also like to thank my extraordinary friends that I have met through my department, as they share in my adversity and are patient with me when my focus needed to be redirected to research. Kayla Cordell has been a supporting pillar of my undergraduate experience and beyond. She builds me up, and encourages me to take on larger challenges. I cannot express enough how meaningful the people in my life are to me, and without them I would be less human.

NOMENCLATURE

gp 156	Gene Product 156
gp157	Gene Product 157
ts	Temperature sensitive
wt	Wildtype
PFU	Plaque Forming Units
MOI	Multiplicity of Infection
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
LB	Lysogeny Broth
TBE	Tris Borate EDTA
LPS	Lipopolysaccharides
CHAP	Cysteine, histidine-dependent amidohydrolases/peptidases
PPM	Parts Per Million
OD	Optical Density
SOC	Super Optimal Broth with Catabolite
PCR	Polymerase Chain Reaction
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis.
MW	Molecular Weight

CHAPTER I

INTRODUCTION

Introduction to Phage Entry into Bacterial Cells

The purpose of this study is to determine lysis capability of a protein product of a possible tail lysin gene, gp156, which is integral to the inject of phage K's DNA into a bacterial cell. Phage entry is a dynamic process, with multiple steps that can go awry. Through field founding experiments with virus T4, a model phage at the time, phage entry is a multistep process, consisting of phage adsorption to the surface of the cell and injection of its DNA. It has been shown that the rate of adsorption to the surface does reach a plateau in high bacterial concentrations, and that it is proportional to temperature. (Stent GS, 1963) First the phage and bacterial cell must have a successful collision so that the phage may be able to bind to the surface receptors reversibly. In the case of T4, the receptor is the lipopolysaccharide (LPS) chains that extend from the outer membrane of *E.coli*, a Gram(-) bacteria. (Stent GS, 1963) Because it is temperature sensitive, and through ionic experiments with virus T1 adsorption, there is reason to believe that the phages change their conformation through interactions with environmental ions into a more receptive form and initially bind reversibly to their bacterial receptors. (Puck TT, et al., 1950) The final phase of adsorption is when the receptor binding proteins in the tail anchor the phage irreversibly to the surface of the cell. (Takeuchi I, et al.,2016) This reaction initiates the injection process. Different viruses differ in this method, specifically in the type of genetic material they inject and the mode of infection they deploy. T4 possess a contractile tail that, upon successful adsorption, under goes configurational change that unsheathes a tail spike the drives through the outer membrane of the cell. The peptidoglycan layer of the cell wall is then exposed

to degrading tail lysins, allowing the rest of the apparatus to penetrate the inner membrane. Finally, its genetic material, dsDNA in the case of T4, is injected into the cytoplasm of the host. The process of injection is the concluding step of phage entry, the initial phase of the viral reproduction cycle.

Characteristics of Phage K

Although there has been intensive research on the nature of phage over the past century, a concise taxonomic convention remains to be desired, as it usually depends on contrasting gene sequences and differences in morphology to discern between phages. The model virus in this study is phage K, a virulent *S. aureus* bacteriophage, which sports some key similarities and differences with the basic T4 *E. coli* bacteriophage. T4 belongs in the *Myoviridae* family under the *Caudovirales* order of phages, meaning that among prokaryotic targeting phages, it sports a contractile tail that exposes a tail spike upon infection. T4's host range is limited to *E. coli,* a Gram(-) bacteria that has a peptidoglycan layer flanked between two plasma membranes, with the outer membrane being peppered with LPS. The T4 capsid is an icosahedron, though slightly prolate, which houses a dsDNA genome of ~170kb. This genome completely codes for T4's replisome, containing a low G+C content, about 34%. T4's genome also includes introns interrupting genes that code for viral enzymes. (Miller ES, et al., 2003) phage K falls in the same order and family as T4, as it also has a prokaryotic host range and sports the same contractile tail structure. An important distinction is that the target host of phage K is *S. aureus,* a Gram(+) bacterium that has a thick layer of peptidoglycan surrounding one plasma membrane layer. This surface contrasts from Gram(-) surfaces with the presence of teichoic acids and tetrapeptides that and mediate peptidoglycan assembly. Tail fibers of bacteriophages that target Gram(+) *S. aureus* phages are known to recognize a combination of aforementioned cell wall elements as a

complex, but the general binding site for phage K receptors is believed to be the backbone of the cell wall teichoic acid. (Takeuchi I, et al., 2016) The capsid protein of all *Caudovirales* phages form the same icosahedral geometry, though phage K's is not prolate. (Appendix C2) The genome of phage K is also large, nearing ~150kb as determined by our laboratory, with a similarly low G+C content. Introns have also been annotated within phage K's genome, save that they are inserted within different genes. (Gill JJ, 2014)

Tail Associated Lysins of Phage K

Another key similarity is that both phages use tail lysins for phage entry. Whereas holins are produced to disrupt the cell membrane and render the peptidoglycan layer vulnerable to endolysins for phage exit, tail-associated lysins are deployed directly onto the cell walls of Gram(+) bacteria from the unsheathed tail spike for phage entry. Sequencing of phage K identifies two such tail lysin genes, the previously characterized gene *157*, and the focus of this study, gene *156*. In our lab, the essential nature of both genes *156* & *157* has been demonstrated via *ts* mutant assays. It is assumed that neighboring genes encode for proteins that are involved in similar processes, which urges investigation further into inherent lysis ability of gp156. Lysins follow a structural pattern where the N-terminus possesses a number out of four possible catalytic sites (muramidase, glucosaminidase, endopeptidase, or amidase) that target different bonds of the peptidoglycan. Usually the C-terminus has a binding domain, specific to its host, that targets major components of the cell wall to orient the N-terminus in close proximity to its target bond (Fischetti VA, 2005). Gp157, however, has no wall binding domain, and the C-terminus possesses the catalytic site, which is predicted as a CHAP domain that hydrolyzes the peptidoglycan layer, and yet demonstrates specificity towards *S. aureus* against other bacterial strains (Paul VD, et al., 2011). Sequencing of gene156 in our lab also reveals a CHAP domain,

and the proximity of gene156 to gene157 in the structural tail module of its gene gives us reason to attempt to classify gp156 as a tail-associated lysin.

CHAPTER II

METHODS

Creating Temperature Sensitive Mutants

Exposing *wt* phage K to nitrosoguanidine concentrations of 5 ug/mL yielded mutant JL42, and exposure to concentrations of 10ug/mL yielded mutant JL201 and JL202. Mutated phage were allowed to grow on a *S. aureus* lawn over night at 30^{o}C on TSA. The next day, using toothpicks, plaques were picked at random and transplanted onto two other *S. aureus* lawns in a grid-format. One plate was incubated at 30^{o}C and another at 40^{o}C. The *ts* mutants of essential genes were identified as plaques that grew on the 30^{o}C plate and not on the 40^{o}C plate. These mutants were then sequenced to identify which gene was afflicted.

Cloning Overview

The cloning experiment is designed to isolate and express a known gene, gene *157*, and the gene in question, gene *156*. (Appendix A2) Cloning consists of amplifying these genes from the phage K genome through PCR with specially designed DNA primers that encode for restriction endonuclease sites. Both the PCR product and the chosen plasmid, in this case pET-Duet1, are then cleaved using the same restriction endonucleases, cleaned, and then ligated together before they are transformed into a competent bacterial strain that will be used to express the construct.

Primer Design and PCR

The primers used in the PCR reaction are meant to flank genes *157* & *156* with restriction sites encoded within. Three pairs of primers have been used in this procedure, each pair matching with a pET-Duet1 vector that will be cleaved with the same endonucleases to generate

sticky ends. One pair of primers is meant to flank gene *156* and allow for a poly-histidine tag, used in purification of the protein, to be placed on the N-terminus of gp156. Primers were provided by Integrated DNA Technologies. With the restriction cleavage sites in "**bold**", the forward 156 HIS primer with a BamHI:

5'-GATCCGTAGCTAATCGAGCTT**GGATCC**GGCAACAGATTAAGAAGC-3'.

The forward 156NOHIS primer, designed to not include a poly-histidine tail and encodes a NcoI site. It reads:

5'-GATCCGTAGCTAATCGAGCTT**CCATGG**CAACAGATAAAGAAGCTAAAG-3'

These primers will both be flanked with a complementary, reverse primer with a NdeI site that reads:

5'-GATCCGTAGCTAATCGAGCTT**CATATG**TTAAATATACACCTCTTCAT-3'

The forward primer for 157HIS with a BamHI site read:

5'-GATCCGTAGCTAATCGAGCTT**GGATCC**GCGTAGAATAAGAAGACCTAA-3'

The reverse complimentary primer of 157HIS with a NdeI site reads:

5'-GATCCGTAGCTAATCGAGCTT**CATATG**TTATTTCTTATCGTAAATGA-3'

The issue arose when designing the 156NOHIS that two methionine residues would have been encoded due to the way the cleavage site and gene *156* would place in relation to one another, skewing translation of the protein. As a response, we elected to preserve the NcoI site and design the primer to change the inherent methionine codon in gene *156*'s mRNA into a proline as previous research shows that the N-terminus of gp157 can be modified and the protein still be functional enough to degrade peptidoglycan. (Paul VD, et al., 2011) For each PCR reaction, 2uL of a given forward primer with 2uL of its designated reverse primer, each at 100uM,was mixed with 1uL of extracted phage K genome as a template, 125uL of PCR Master Mix with OneTaq

DNA polymerase included, and 120uL of water. The total volume of 250uL was then vortexed and dispersed among five PCR reaction tubes in equal parts, and was allowed to react overnight using a BioRad PCR thermocycler. The PCR program used proceeds with 3min 30sec in 95^{o}C, 30sec in 52^{o}C, then 72^{o}C for 3min. The reaction was then held at 95^{o}C for 960sec, then allowed to drop to 72^{o}C for 5min. Afterward, the reaction was maintained at 12^{o}C until extraction. This PCR program was used for both gene *156 & 157*.

Cloning into Plasmid

The chosen vector in this experiment is pet-DuetI, which is capable of being transformed and expressed in *E. coli* and possesses an ampicillin antibiotic resistance. (Appendix A1) A stock of *E. coli* carrying this plasmid was grown overnight and then centrifuged at 8000rpm, 4^{o}C for 10 minutes. The supernatant was discarded and the pellet was suspended within the residual supernatant. The plasmid was extracted and purified using the QIAGEN Plasmid Mini Prep kit. 20uL of PCR insert and plasmid vector were both separately subjected to 1uL of each respective enzyme, 3uL of CutSmart Buffer from NEB Labs, and allowed to react overnight. The next day, the insert was purified of excess protein and DNA via QIAGEN's PCR Purification kit, and the vector was purified via gel extraction. For the gel extraction, a 1% Agarose Gel was made mixing 50ml of TBE buffer with .5g of agarose and 5uL of GelRed Stain purchased from Biotium and left to dry in a mold. The gel was subjected to electrophoresis, via Fisher Scientific power source, and allowed to run for 45 minutes. It was then visualized with UV light. Once the correctly cut vectors were exposed, they were then cut out of the gel with sterile razors and subjected to the QIAGEN Gel Purification Kit to remove the agrose environment from the plasmid digest. Once both the digested insert and vectors were purified, they were then combined in a ligation reaction, containing 10uL of vector (35 ng/uL), 10uL of insert (300ng/uL), 2uL of

T4 Ligase, and 3uL of T4 Ligase Buffer from NEB Labs, and allowed to react at room temperature overnight.

Transformation

The ligated construct was then transformed into *E. coli* strain DH5a. After thawing DH5a (NEB) on ice, 5uL of each construct was pipetted into their own eppendorf tubes with 25uL DH5a inside and gently mixed and allowed to sit on ice for 30min. The tubes were then exposed to a 42ºC water bath for 30 seconds to heat shock the bacteria. The reaction rested on ice for 5 minutes, and then incubated in 950uL of SOC solution for an hour at 37ºC. Once the bacterial cells were allowed time to recover, the entire reaction was then plated on one plate of LB with ampicillin, at 100ug/mL, and allowed to grow overnight at 37ºC. The next day, isolated colonies were inoculated onto another set of plates with TSA and ampicillin and were also allowed to grow overnight. Isolated colonies from these plates were then grown up in TSB with ampicillin and then underwent plasmid purification, as mentioned above, and digested with their respective enzymes to be subject to gel electrophoresis to confirm that the insert was ligated properly.

Cesium Gradient Phage Purification

Purifying phage is a foundational protocol in this endeavor, as this allows us to calculate mutant and *wt* titers and extract genomes using pure virions. First we grew 50mL cultures of *S. aureus* strain Newbould 305 (Gill Lab Strain #1007) to an OD reading of 0.5 at 550nm at 30ºC. Keeping the temperature is important, as it allows both *wt* and *ts* mutant phage to infect bacterial cells successfully. Once the desired OD was reached, the bacterial cells were infected at an MOI of 5, and left overnight. The next day, the lysate was then chloroformed and centrifuged at 8000 rpm, 4ºC for 10 min to rid of any leftover living cells and cellular debris. The supernatant was preserved, and the chloroform and debris pellet was discarded. Next, a cesium density gradient

was created, first by adding 2mL of 10% Sucrose to the bottom a plastic ultracentrifuge tube, then 2mL of 1.4 g/cm^3 CsCl solution to the very bottom of the tube, then finally 2mL of 1.6 g/cm^3 CsCl solution to the bottom. The saved supernatant from the lysate, that contains the phage particles, is then added to the top of the tube until the tube is completely full. Using a Beckman L8-70M Ultracentrifuge with a SW41Ti rotor, these tubes were then centrifuged at 35000 rpm for an 1.5 h, 4 ºC. The tubes were viewed in a dark room over an LED light, which revealed a cyan, opaque band of purified phage between the 1.4 and 1.6 g/cc cesium phase. (Appendix D2) These entities were extracted by piercing the side of the tube with a needled syringe and then transferred to a dialysis membrane. The membrane was subject to 1M NaCl Lambda solution for one day in the cold room to diffuse the excess cesium out of the phage fraction. The membrane was moved to fresh lambda solution the next day, and after diffusing the salt out of the phage fraction, the now pure phage fraction was saved for genome extraction and titer.

Phage Titer

After being purified through a cesium gradient, the phage were then titered, which is a technique to determine the number of plaque-forming phage particles in one mL of stock. This could also be done with raw lysates. The stock was serially diluted into 4 100-fold dilutions by aliquoting 10uL of stock into one mL of Lambda dilution, then 10uL of that solution into another mL of Lambda dilution, and repeated for another two times. A bacterial lawn of *S. aureus* strain 1007 was then prepared and spotted with 10uL drops from each dilution. These lawns were then incubated at 30ºC, 37ºC, and 40ºC to test for temperature sensitivity. The next day, the number of plaques were recorded for the most dilute spot, and then calculated to determine plaque forming units (PFU) per mL by dividing the number of plaques in a given spot by the product of

the volume spotted in milliliters (.01 mL) and the dilution factor from the dilution that made the plaque. It is accepted that it only takes one phage to create a plaque by way of reproducing a large number of phages and then lysing to release the progeny to infect more, therefore 1 PFU is assumed to be 1 active phage in a solution.

Stability Assay

25mL of *S. aureus* were allowed to grow to an O.D of .5 at 30^{o}C in individual 250mL flasks. Once the O.D was reached, they were infected with concentrated stock of *wt* and mutants of phage K. Each flask was allowed to undergo complete lysis overnight. The next day, the lysate was subject to 200uL of chloroform for half an hour and then centrifuged at 8000rpm at 4^{o}C for 10 minutes to pellet cellular debris and kill possible surviving colonies. The supernatant above the phase separation was extracted and transferred to its own conical tube. A water incubator was conditioned to 50^{o}C and 1mL of each phage mutant lysate was transferred into one set of designated eppendorf tubes. The lysate was titered at the initial hour while the set of eppendorf tubes were incubated in the 50^{o}C water bath. They were removed from the bath and then titered after 24hrs.

Preparing SDS-PAGE Gel

First, 50uL sample of phage particles were boiled in 50uL of ultra-purified water for 5 minutes. For every 15uL of phage sample, 1uL of DNAase was added and allowed to incubate in 37^{o}C for 30min. Using a premade SDS-PAGE Gel provided by ThermoFisher Scientific, three wells were loaded with 20uL, 15uL, and 5uL of denatured phage sample. SDS-Sample Buffer was made from 100uL of SDS Running Buffer and 10uL Dithiothreitol (DTT) 1M. 5uL of the resulting solution was added to each of the three sample volumes. The wells were then flanked by a protein standard provided by Thermo Fisher Scientific. The gel was then subjected to

electrophoresis for 2hrs at 100V. Afterwards it was soaked in a fixing solution, replacing the solution every 30minutes. After the second soak in fixing solution, the gel was then soaked with distilled water in a stain box for three increments of 10mins. Afterward, the gel was left to soak overnight in a tinfoil wrapped box with Sypro RubyRed PAGE Gel Stain. The next day, the gel was rinsed with water and then imaged by a Gel Imaging System. ImageJ was the program used to invert the colors in the photo taken. SnapGene was used to determine the molecular weight of our hypothetical protein and guided us in predicting its location on the gel.

Potassium Release Assay

S. aureus was allowed to grow in 50mL batches in TSB to an O.D of .5 at 30^{o}C. They were then centrifuged at 8000rpm at 4^{o}C for 10min. The supernatant was discarded, and the cells were suspended in potassium phosphate buffer at half the original volume. (Leavitt J, et al., 2013) The cells were then centrifuged with the same conditions a second time, then suspended to 15mL with potassium phosphate buffer. The 15mL volume was then split into three 5mL volumes and placed on ice. Potassium Ion Standard buffers were made by serially diluting 1000ppm potassium stock, purchased from VWR, to make three different solutions, 100ppm, 10ppm, and 1ppm, and were measured with a potassium ion electrode before each infection. *Wt* and mutant phage K were grown and cesium purified, and with a known titer, infected their own 5mL bacterial stock at 30^{o}C at an MOI of 20. The electrode measured the release of potassium ions, in a similar fashion to a pH reader, that leaked from the cell as a result of DNA injection. Recordings were allowed to proceed until the peak of potassium release passed, or until a 30min limit was reached.

CHAPTER III

RESULTS

Temperature Sensitive Mutants

Wt phage K was exposed to nitrosoguanidine to yield mutants. Exposing the same mutated plaque to two different temperatures allowed us to screen for mutants in genes that were essential to a phages survival. Plaques that would not survive in the higher temperature but grow in the lower would have mutations in genes that coded for necessary proteins. This is likely due to the misfolding of the protein product, and increasing the temperature would exploit the change of amino acid sequence to denature the product faster than it would normally. The plaques on the 30^{o}C lawn that corresponded to an empty space on the 40^{o}C lawn were extracted and sequenced by our lab. (Figure 1) The genes that were affected by the mutagen were random, but our lab happened upon a single point mutation in gp157, designated as JL201, converting serine625 to a leucine. Single point mutations were also found through sequencing in gp156, where JL202 converted glutamine122 to a lysine, a change from a negatively charged residue to a positive charge, and JL42 converted a proline203 to a leucine. (Appendix A2)

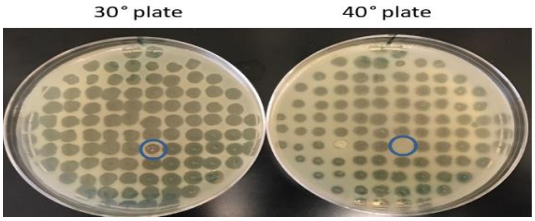

Figure 1. In a gridded fashion, mutated phages were transferred from a single plaque onto two new plates, each exposed to different temperatures. Plaques that grew on only the 30^{o}C plate were mutants of essential genes.

Cloning of gp156

After transformed colonies were selectively grown on antibiotic plates, they were inoculated in ampicillin LB (100ug/mL). Putting them in the through plasmid purification and running their restriction digest through a gel allowed us to screen for correct transformation. (Figure 2) Initially, to investigate the lysis ability of gp156, our intention we to clone gene156 into a shuttle vector and transform it into competent *E.coli,* purify it, and perform a zymogram on the protein to see if it degraded peptidoglycan without being attached to a virion. However, after transforming our construct into DH5a and running a restriction digest, they appeared to be empty.

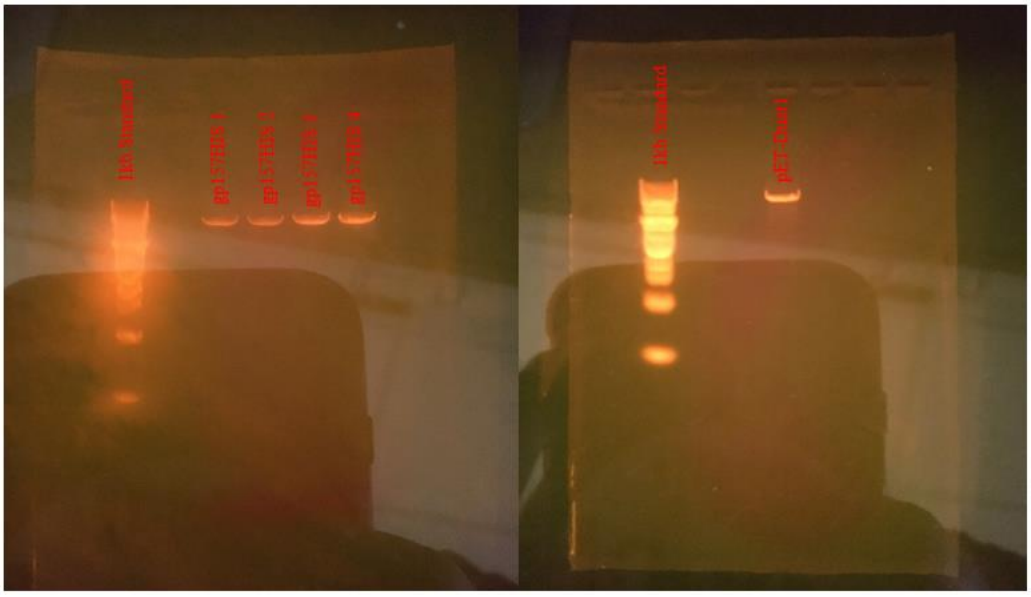

Figure 2. The image to the left is a restriction digest of our construct from the DH5alpha colonies that grew on selective plates. The image to the right is the same digest of empty pET-DuetI. Cloning gene156, both with and without HIS tags, resulted in similar restriction digest gels.

SDS-PAGE Results

After visualizing the SDS-PAGE gel of *wt* phage K, we highlighted masses of key proteins that make up the virion of bacteriophage 812, a highly similar virus to phage K, which

are listed in Table 1, as well as the mass of gp156 on the gel. (Eyer L, et al., 2011) Upon visualizing the gel, a dark band can be seen around the area that gp156 is predicted to fall in. (Figure 3). Using the molecular weight predicted by SnapGene, we could make a prediction of it would fall on the gel. However, through preliminary mass spec data, there are a few other hypothetical proteins (gp92, gp96, gp95) that are of similar MW. (Appendix B1)

Table 1. Line Assignments to SDS-PAGE Gel of phage K

Line Assignment	**Gene Product**	**Molecular Weight in kDa**	**Function**
1(1st Intense Band)	Gp150	116.2	Hypothetical Protein
2	Gp155	96.1	Phosphodiesterase
3	Gp138	70.2	ATPase
4	Gp176	64.2	Portal Protein
5 (2nd Intense Band)	Gp64	56.1	Nicotinamide Phosphoribosyl Transferase
6 (3rd Intense Band)	Gp173	51.2	Major Capsid Protein
7	Gp151	39.2	Tail Protein
8 In red	Gp156	34.6	Supposed Tail-Lysin Cysteine Protease
9	Gp170	33.7	Hypothetical protein
10	Gp154	29.5	Hypothetical protein
11	Gp175	28.6	Capsid Protein
12 (4th Intense Band)	Gp70	25.8	Hypothetical protein
13	Gp205	23	Hypothetical protein
14	Gp165	15.6	Hypothetical protein

Note: Line assignments are displayed in the SDS-PAGE Gel Figure 3.

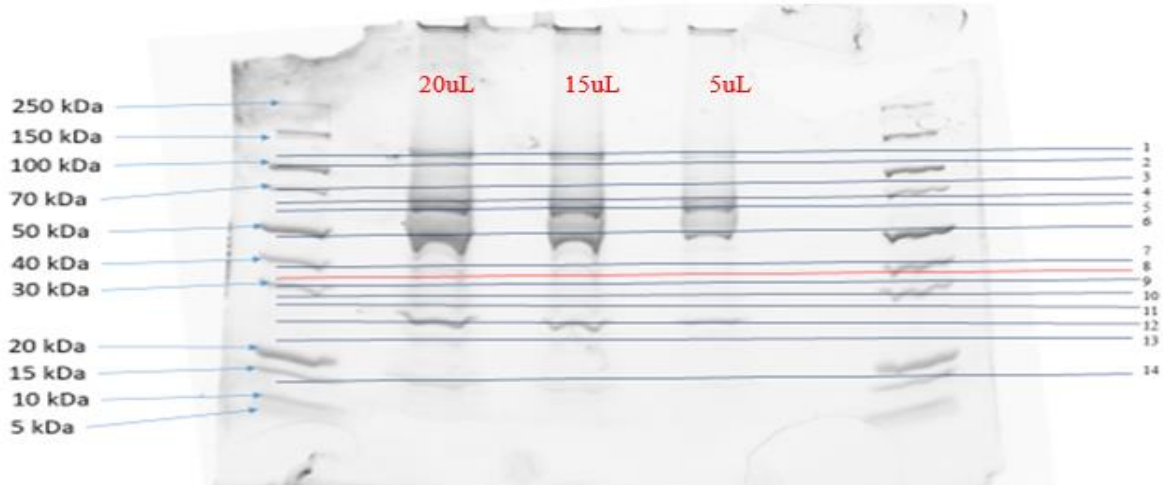

Figure 3. The first and last wells in this SDS-PAGE Gel image contain a protein standard of proteins with known weights. The middle three wells, from left to right, contain 20uL, 15uL, and 5uL of denatured *wt* phage K virion with 5uL of SDS Running Buffer in each. Line assignments are numbered and described in Table 1, with line 8, in red, highlighting the position of a possible gp156 band. Refer to the appendix to view the gel without the line assignments.(Appendix C1)

Stability of Mutants

After 24 hours the titer reduced 10-fold in 201. (Figure 5) In gp156 mutant 42, it is also reduced 10-fold, and 202 is almost completely gone, reducing in four orders of magnitude. *Wt* also undergoes a 10-fold reduction in titer. The lysates of mutant 202 after 24hr exposure to the 50^{o}C bath were visualized by TEM and compared to *wt*. Though there were complete capsid proteins and tail sheaths, in most instances they were apart from each other. There would also be record of an occasional virion with capsid protein and tail sheath attached, but an incomplete base plate, as depicted in the TEM photographs. (Figure 4)

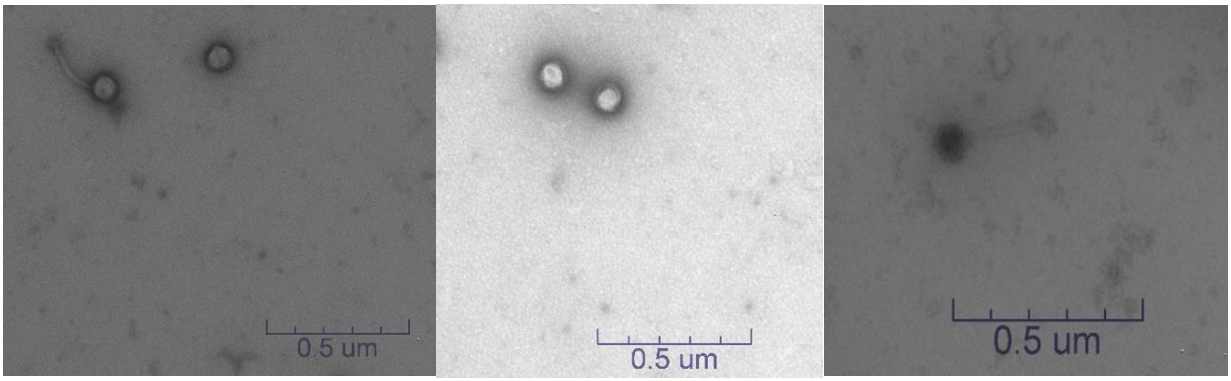

Figure 4. These three images are TEM photographs at 25000 power. The left and center images are pictures of mutants JL202. Disassembled virions that appear as only head or tail proteins were more common than virions with only head and tail proteins. The right image is *wt* phage.

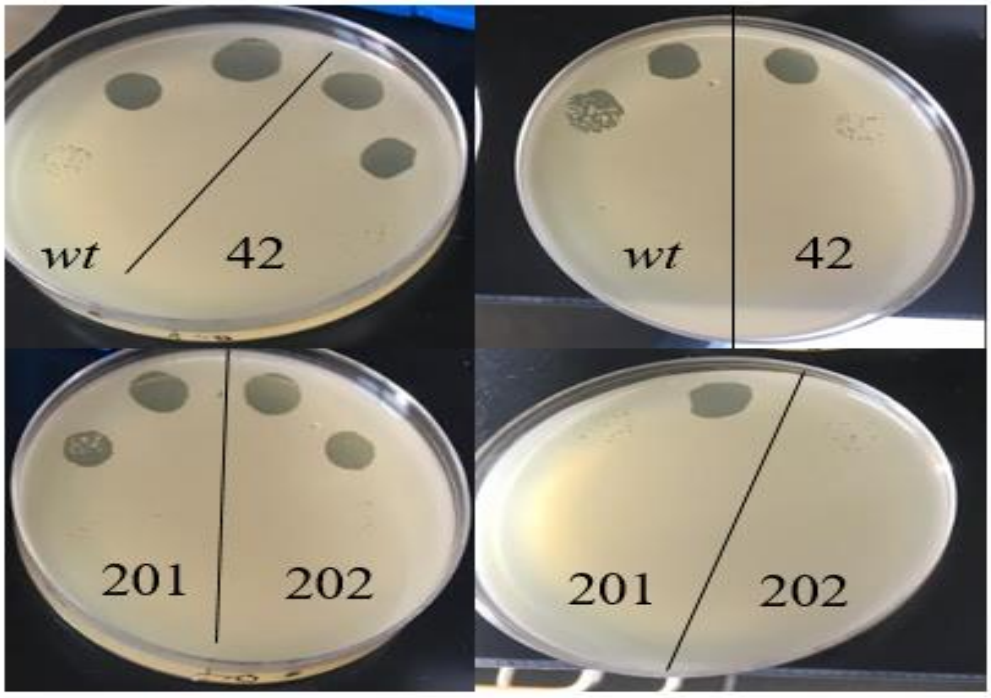

Figure 5. Phages were serially diluted into dilutions of 10^{-2}, 10^{-4}, 10^{-6}, 10^{-8}. 10uL of each dilution was then plated next to one another and incubated in 30^{o}C overnight.

Potassium Release Data

Because potassium ions escape the cytoplasm during DNA injection into a bacterial cell, potassium release is directly related to the amount of DNA being transduced and the speed of the injection process. By tracking the amount of potassium ions that are released into the buffer in real time, we can measure the speed at which *wt* and mutant phage K can infect. (Figure 6a,6b) From this data, at both temperatures permissive and non-permissive temperatures, we observe that all three types of mutants infect their host at a much slower rate than *wt*. JL42 maintains to be the slowest out of all mutants, and infects slower at non-permissive temperature. Infection by JL201 and JL202 behave similarly as their trends tend to overlap at non-permissive temperature. During permissive temperature measurements, JL201 exhibits a flat slope initialized by a brief 1minute spike, which is unlike the other neighboring trends and, even more so, past potassium release data collected in this laboratory. (Appendix D1)

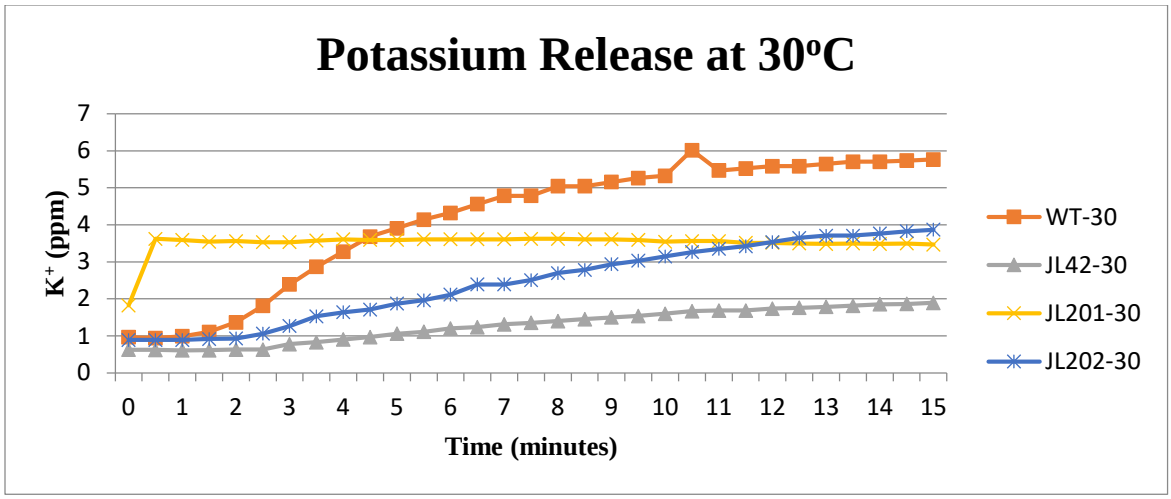

Figure 6a. Potassium Release curve of *wt* and *ts* mutants generated using data from infection at 30ºC.

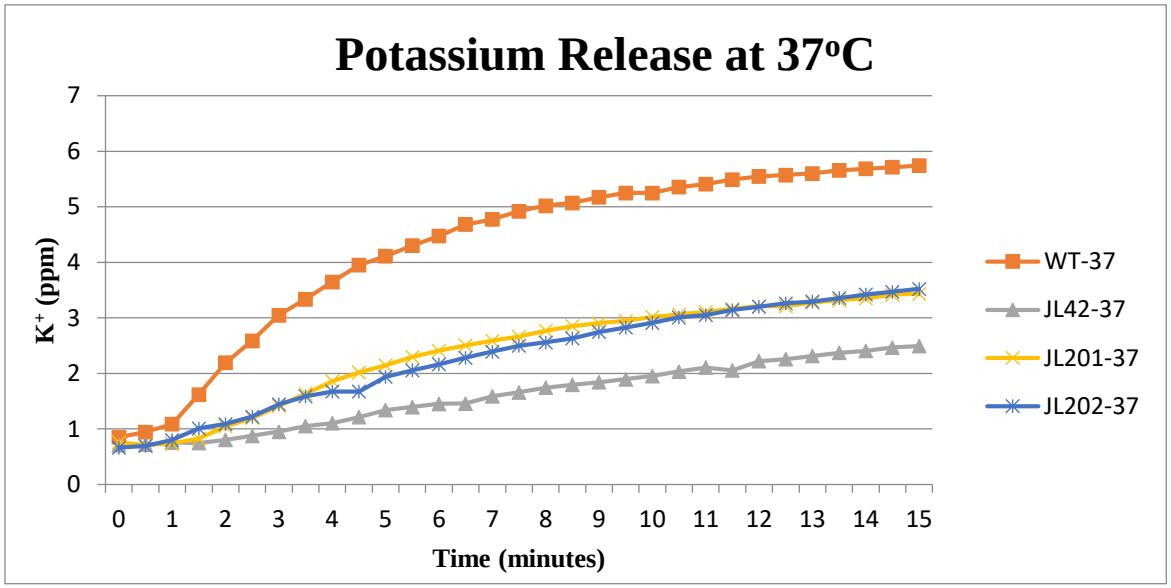

Figure 6b. Potassium Release curve of *wt* and *ts* mutants generated using data from infection at 37ºC.

CHAPTER IV

CONCLUSION

Initially, this study sought to determine the lytic abilities of gp156, a hypothetical tail-associated lysin. In order to accomplish this, we looked toward cloning gene *156* into a vector, while simultaneously cloning gene *157*, and expressing it apart from the rest of the other phage processes. However, these attempts to clone gene *156* and gene *157* were unsuccessful. Human error following these cloning protocols could contribute to unsuccessful cloning, or perhaps our materials were expired and mislabeled. However, the foreign DNA of a Gram(+) virus entering Gram(-) *E. coli* could be causing some unforeseen interruptions in regulatory pathways and could be toxic. It is unlikely that the gp156 translated in the cytoplasm are degrading the cell wall because of the inner membrane layer that separates the two. The aforementioned protocol in the methods section was written using the most recent cloning attempt, but there were four other attempts using different vectors before this one. It could be possible that the competent strain is the issue and maybe changing this variable would yield success. Further research is required to perfect this method. Future directions would be to purify this protein and run a zymogram with autoclaved *S. aureus* to determine inherent lysis ability.

This study did, however, find that gp156 and gp157 are essential for correct phage replication. Through discovery of *ts* mutants, gene156 and gene157 yielded a single plaque at permissive temperature, but when exposed to non-permissive temperature, there was no plaque observed, indicating an error in virion processing. We found two possible explanations for why there is a difference observed through the stability and potassium release assays.

After running an SDS-PAGE gel with denatured phage K protein, we noticed a slightly darkened band in at the predicted position of 34.6 kDa. It should be noted that this gel is the first to distribute and display phage K and its proteins. TEM pictures of our stability assays revealed the presence of incomplete virions, where morphogenesis of the head and tail constituents proceeded to completion, but were never combined together. Baseplates were also absent entirely. This evidence suggests that gp156 and gp157 play a role in structural integrity.

Through potassium release data, we were able to measure the amount of potassium ions present in a solution of infected bacteria in real time at the moment of infection. Measurements of standards allowed us to compare these readings and generate a standard curve. From the trends of these curves, it is shown that at non-permissive temperature, potassium release of these mutants, compared to potassium release of *wt* phage K, is slowed. This trend holds constant when infection is allowed to proceed at permissive temperature. Slower potassium release correlates to a smaller amount of viral DNA injecting into a cell over a defined period of time. Therefore, our data shows that genes156 and 157 are not only essential for structural integrity of the virion, but are also vital for proper infection at the beginnings of the phage cycle.

It should be of note that potassium release data from JL201 has been consistently aberrant. As shown in in the appendix, potassium release data from last year demonstrated faster DNA injection compared to *wt*. (Appendix D1) Our most recent data depicts a slower rate of injection, but at 30^{o}C it reached a plateau. This could be explained by the differences in condition of the host being grown at different temperature that effect the phage entry process, but our data encourages more data to be collected.

Future directions of this research would be to sufficiently clone these two genes and run their respective gene products through zymograms with and a part from each other, following the

protocol conducted by Paul VD, et al. It would also be beneficial to the field of phage biology to image these proteins through crystallography and cryogenic electron microscopy techniques. Once these experiments have been conducted and proofed, treatment experiments on live organisms infected with *S. aureus* could provide data and direction of using these proteins as antibiotic alternatives.

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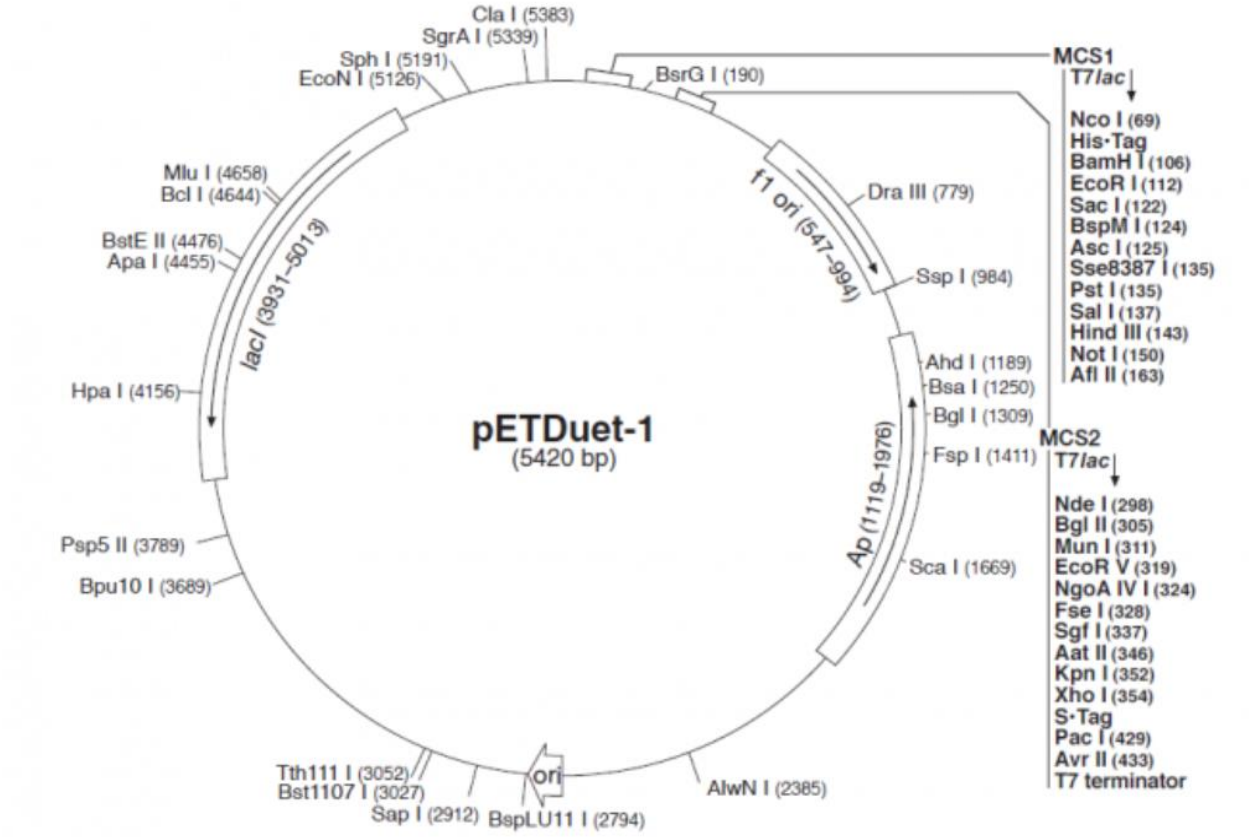
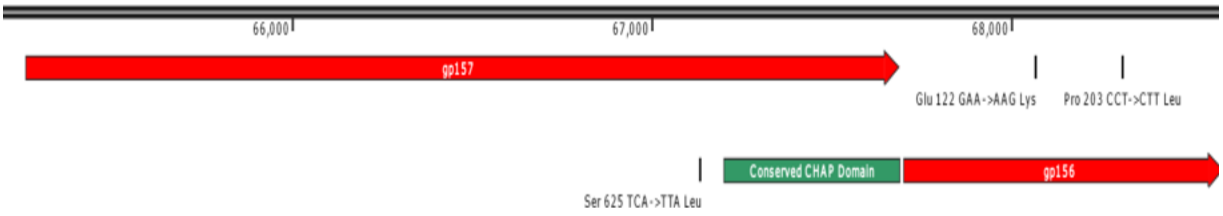
APPENDIX A



A1. pETDuet-1 was used in an attempt to clone genes *156 & 157*



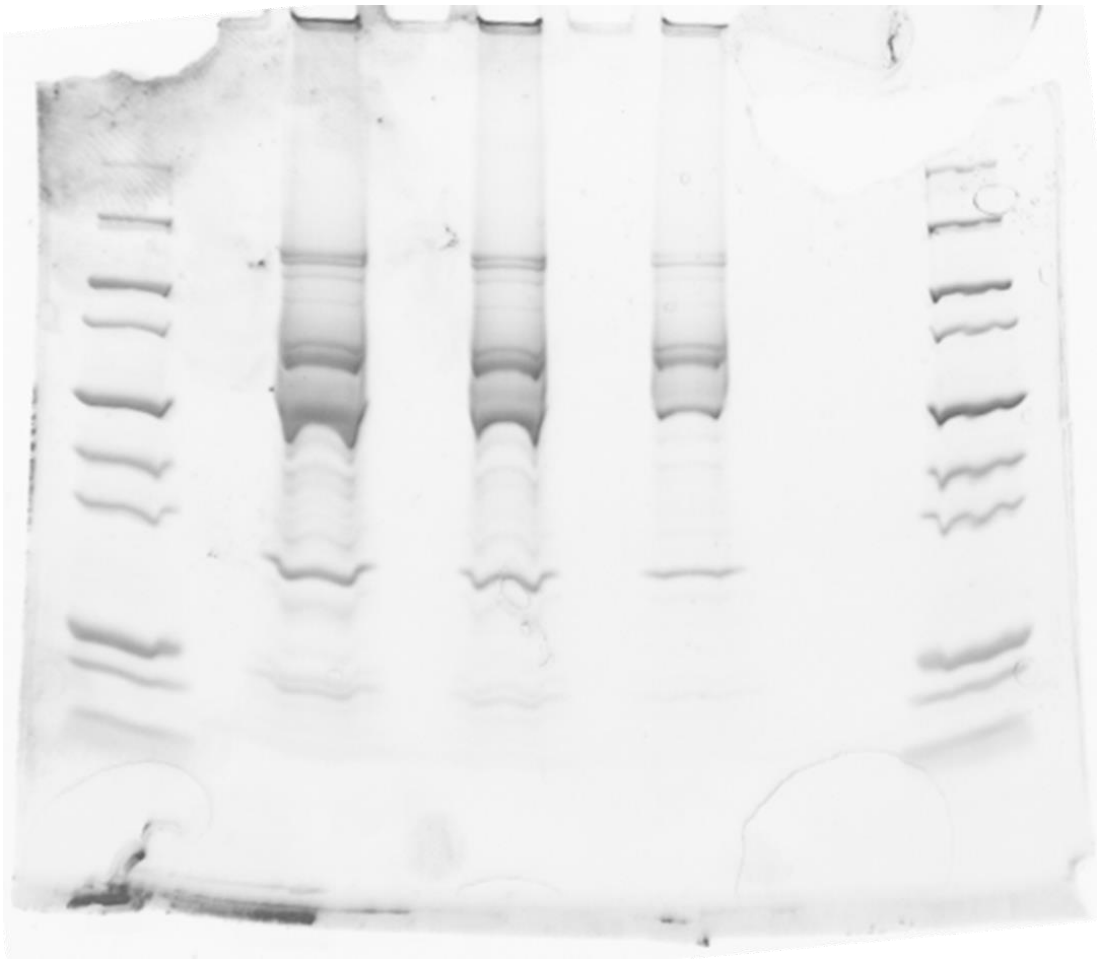
A2. Location of genes *156* & *157* in the genome with listed mutations

APPENDIX B

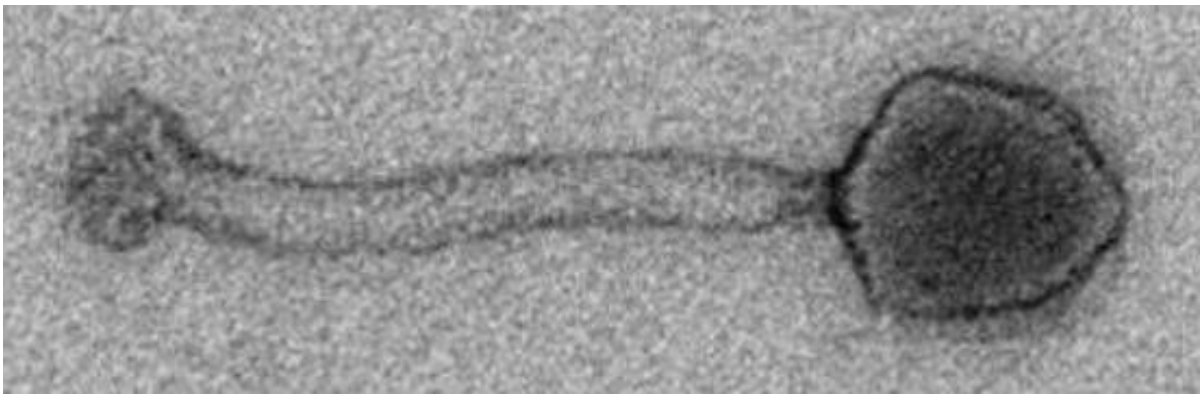
Protein IDs+AHA1:AH39	Unique peptides	Sequence coverage [%]	Mol. weight [kDa]	Score			genebank
tr|Q6Y7R2|Q6Y7R2_BPPGK	111	75.3	143.83	323.3	Putative tail tape measure protein	ORF55	PhageK_108
tr|Q6Y7Q2|Q6Y7Q2_BPPGK	137	95.1	129.12	323.3	Unknown?	ORF65	PhageK_118
tr|Q6Y7Q4|Q6Y7Q4_BPPGK	65	70.9	116.28	323.3	Unknown?	ORF63	PhageK_116
tr|Q6Y7Q9|Q6Y7Q9_BPPGK	49	60	96.095	323.3	Putative phosphodiesterase	ORF58	PhageK_111
tr|Q6Y7R1|Q6Y7R1_BPPGK	50	70.4	91.236	323.3	Putative CHAP domain protein	ORF56 (gp157)	PhageK_109
tr|Q6Y7Q1|Q6Y7Q1_BPPGK	65	85.9	72.532	323.3	Putative carbohydrate-binding domain	ORF66	PhageK_120
tr|Q6Y7R8|Q6Y7R8_BPPGK	84	94.9	64.49	323.3	Putative tail sheath protein	ORF49	PhageK_100
tr|Q6Y7S6|Q6Y7S6_BPPGK	42	57.7	64.068	323.3	Putative portal protein	ORF41	PhageK_090
sp|Q6Y7S3|CAPSD_BPPGK	50	89.6	51.237	323.3	Major capsid protein	ORF44	PhageK_093
tr|Q6Y7P9|Q6Y7P9_BPPGK	40	82.8	50.424	323.3	Putative receptor binding protein	ORF68	PhageK_122
tr|Q6Y7S1|Q6Y7S1_BPPGK	24	81.5	33.716	323.3	Unknown?	ORF46	PhageK_096
tr|Q6Y7R9|Q6Y7R9_BPPGK	19	66.5	31.767	323.3	Unknown?	ORF48	PhageK_098
tr|Q6Y7T1|Q6Y7T1_BPPGK	18	56	30.663	323.3	unknown?	ORF36	PhageK_084
tr|Q6Y7S5|Q6Y7S5_BPPGK	26	91.1	28.642	323.3	Putative prohead protease	ORF42	PhageK_091
tr|Q6Y7M4|Q6Y7M4_BPPGK	38	99.5	23.199	323.3	Unknown?	ORF95	PhageK_150
tr|Q6Y7Q3|Q6Y7Q3_BPPGK	25	99.4	19.239	323.3	Unknown?	ORF64	PhageK_117
tr|Q6Y7M3|Q6Y7M3_BPPGK;tr|Q2G2K2|Q2G2K2_STAA8	17	69.4	17.848	323.3	Putative bacterial adhesin/Ig-like prot	ORF96	PhageK_151
tr|Q6Y7R7|Q6Y7R7_BPPGK	16	93	15.925	323.3	Putative tail tube protein	ORF50	PhageK_101
tr|Q6Y7Q8|Q6Y7Q8_BPPGK	16	52.9	29.343	280.1	unknown?	ORF59	PhageK_112
tr|Q6Y7Q5|Q6Y7Q5_BPPGK	18	52.6	39.209	274.9	Putative baseplate protein	ORF62	PhageK_115
tr|Q6Y7Q6|Q6Y7Q6_BPPGK	15	82.9	26.583	231.9	Unknown?	ORF61	PhageK_114
tr|Q6Y7S2|Q6Y7S2_BPPGK	12	52.3	34.16	197.6	Unknown?	ORF45	PhageK_095
tr|Q6Y7Q7|Q6Y7Q7_BPPGK	13	64.9	19.983	197.3	Unknown?	ORF60	PhageK_113
tr|Q6Y7R5|Q6Y7R5_BPPGK	6	35.9	12.252	152.3	Unknown?	ORF52	PhageK_105
sp|Q2G0N0|EFTU_STAA8	12	44.7	43.103	139.1	**Elongation factor Tu**		Host
tr|Q6Y7S0|Q6Y7S0_BPPGK	10	44.7	23.76	108.8	Unknown?	ORF47	PhageK_097
tr|Q6Y7T6|Q6Y7T6_BPPGK	16	46.3	54.74	86.23	Putative endolysin	ORF30/ORF32	PhageK_071
tr|Q6Y7N5|Q6Y7N5_BPPGK	7	81.7	12.384	85.39	Unknown?	ORF82	PhageK_137
tr|Q6Y7N4|Q6Y7N4_BPPGK	6	58.5	12.059	82.02	Putative thioredoxin-like protein	ORF83	PhageK_138
tr|Q6Y7R0|Q6Y7R0_BPPGK	12	41.7	34.611	76.8	Putative cysteine protease	ORF57 (gp156)	PhageK_110
tr|Q6Y7S4|Q6Y7S4_BPPGK	5	21.5	35.741	44.87	Unknown?	ORF43	PhageK_092

B1. This table is preliminary mass spec data gathered by our lab. Though it still needs to endure more vetting, as of now, it is entirely possible that both gp156 and gp157 are associated with virion structure. Dark fields correlate to phage protein, and light gray to bacterial proteins.

APPENDIX C

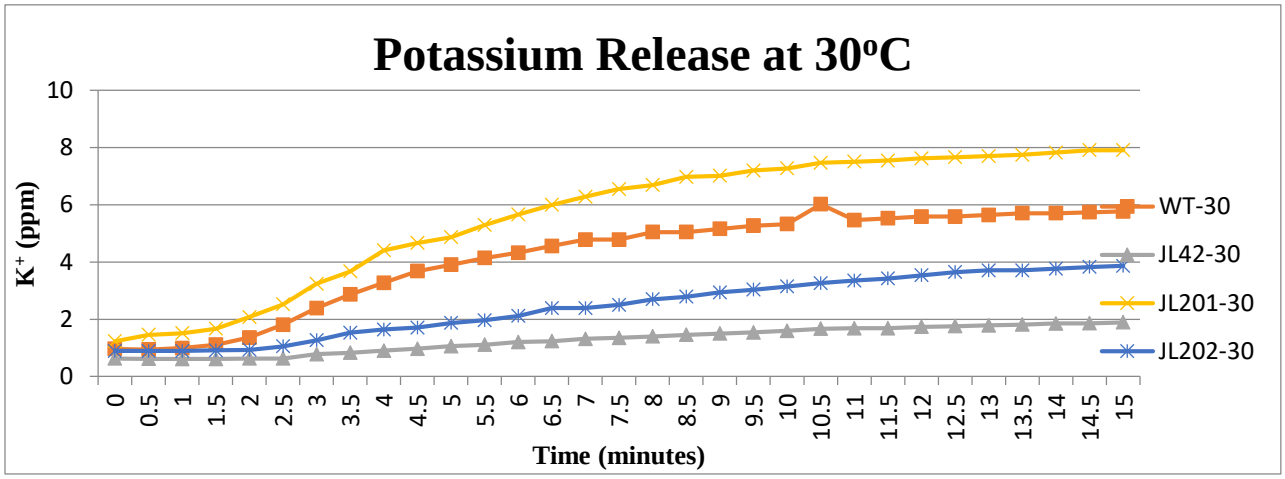


C1. Original SDS-PAGE gel image without line assignments

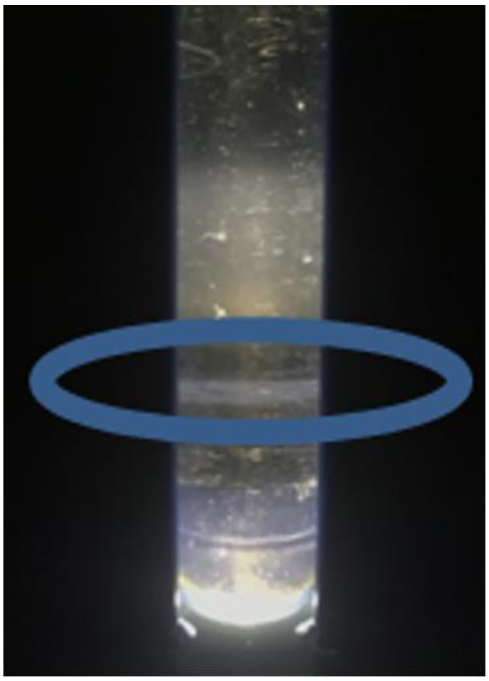


C2. Close up image of phage K taken by our lab.

APPENDIX D



D1. Aberrant Potassium Release Data of JL201 Oct.2018



D2. Cesium Gradient Purification of phage K